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Method

Multiplex locked nucleic acid probes for analysis of hepatitis B virus mutants using real-time PCR

Zhen Sun [a,b], Linfu Zhou [a,1], Haiyan Zeng [c,1], Zhi Chen [a,b,*], Haihong Zhu [b]

[a] *Institute of Infectious Disease, First Affiliated Hospital, Medical College, Zhejiang University, 79 Qingchun Road, and Key Laboratory of Infectious Disease, Ministry of Public Health of China, Hangzhou, Zhejiang 310016, Peoples' Republic of China*
[b] *Experimental Center for Molecular and Cellular Biology, Medical College, Zhejiang University, 353 Yan'an Road, Hangzhou, Zhejiang 310031, Peoples' Republic of China*
[c] *Department of Veterinary Medicine, Qinghai University, 97 Ningzhang Road, Xining, Qinghai 810016, Peoples' Republic of China*

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Abstract

Current methods of detecting hepatitis B virus (HBV) mutations are time consuming, labor intensive, and not suitable for screening large numbers of samples. A multiplex real-time PCR approach presented in this article is a hepatitis B virus quantification method that employs the SYBR Green I dye in conjunction with wild-type HBV sequence-specific locked nucleic acid (LNA) probes. The three short LNA probes distinguished the wild-type strain or three groups of mutants (rt173, rt180/rt181, and rt202/rt204) depending on perfect-match hybrids or mismatch within one template simultaneously. Primers labeled with quencher minimized the background signals. This sensitive approach could quantify 10^2 copies of HBV virus, and as low as 1% mutants among 10^4 copies of wild-type HBV could be identified. The technique is handy and convenient, requiring only 3.5 h to analyze 30 hepatitis B surface antigen-positive serum samples. The HBV isolates were confirmed by direct sequencing. Our data indicate that real-time PCR with SYBR Green I dye is a reliable, rapid, and convenient technique for HBV quantification. Furthermore, by incorporating fluorescent LNA probes, this technique becomes handy in identifying and classifying mutations in the HBV *polymerase* gene. Being sensitive, specific, accurate, rapid, and convenient in nature, this technique could be a suitable diagnostic tool with wide application particularly in cases in which large volumes of clinical samples are handled.

Keywords: HBV mutant; Locked nucleic acid; Multiplex real-time PCR

Hepatitis B virus (HBV) is a partially double-stranded DNA molecule virus that replicates through an RNA intermediate by reverse transcription [1]. The reverse transcriptase responsible for this aspect of the replication cycle lacks proofreading function and, therefore, single base substitutions to the genome are common. Moreover, prolonged therapeutic interventions of antiviral therapy with adefovir, emtricitabine, lamivudine, and/or telbivudine result in drug-induced gene mutations, which appear as drug resistance [2–8], and a number of mutations affecting almost exclusively amino acids in the B domain of the HBV polymerase (rtV173L, rtL180M, and rtA181TV in domain B and rtM204SV/I in domain C) have been described. Thus, the ability to detect these mutants is essential to further our understanding of the natural history of HBV and its response to antiviral therapy and immunoprophylaxis.

The specificity of mutation detection in antisense oligo (ASO) hybridization is based on the difference in melting behavior of perfectly matched duplexes with the target probe and single mismatches [9]. The greater the difference between the melting temperature (T_m) of the matched versus mismatched duplexes (ΔT_m) is, the better the oligo probe discriminating power is [9]. Since the stability difference between a perfectly matched duplex and a mismatched duplex can be as small as 0.5°C, the discrimination between two DNA sequences that differ by only 1 base is difficult. This ΔT_m is dependent upon the length of the probe, the type

* Corresponding author. Fax: +86 571 87217044.
E-mail address: chenzhi@zju.edu.cn (Z. Chen).
[1] These authors contributed equally to this work.

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of mismatch, and the neighboring nucleotides. The longer the ASO probe, the smaller the effect of a single-base mismatch on overall duplex stability. The differential stability of a matched versus mismatched duplex is the main limitation of the use of oligonucleotide hybridization for detection of single base substitutions. Oligonucleotide probes containing locked nucleic acid (LNA) residues have shown strong affinity for their complementary targets [10]. Because of their high thermal stability when hybridized to DNA, LNA can be designed such that they have a shorter length while keeping a high T_m, hence enabling probe hybridization to occur during the PCR annealing step. Recently, several reports have documented the high discriminatory power of oligonucleotides containing LNA to differentiate between match and mismatch duplexes [11–13]. Here, we describe a five-color quadruplex real-time PCR platform for virus DNA quantification and mutant identification based on SYBR Green I dye in conjunction with LNA probes. The target DNA (164 bp) within five mutants was amplified with quencher primers, and wild/mutant sites assembled into three groups were discriminated with three LNA probes, respectively. The total viral DNA level and wild-type proportion of total viral DNA were calculated according to the relevant standard curves (SYBR Green I dye for total viral DNA level, probes 173, 180, and 204 for wild-type proportions of sites rt173, rt180/181, and rt202/204, respectively), while mutant proportions of total viral DNA were assayed using wild-type probes compared to total viral DNA level.

Results

LNA probes for mutant detection

By incorporating LNA residues into synthetic oligonucleotides, probes can be made shorter while maintaining a high T_m, allowing several probes to hybridize on the same fragment for a particular site discrimination assay. The probe hybridized only to its complementary wild-type PCR products and not to the mutant PCR products (Fig. 1A).

Multiplex real-time PCR assay with LNA probes for the detection of the mutant control plasmids

To assess the performance of probes containing LNA as tools for multiple-site mutation discrimination in a five-color multiplex 5′-nuclease assay, we developed an assay for detection of three groups of mutations involved in HBV drug resistance, with respect to the antiviral drugs adefovir, emtricitabine, lamivudine, telbivudine, etc. To demonstrate that the multicolor assay specifically distinguishes the wild-type and three groups of mutations, differentially labeled probes containing LNA were designed using the strategy described under Materials and methods. The three probes were designed complementary to the wild-type strain and labeled with 6-carboxy-4′, 5′-dichloro-2′,7′-dimethoxy fluorescein (JOE) (rt173), Cy3 (rt180/rt181), or Cy5 (rt202/rt204) (Table 1). Total PCR amplicon was monitored by measuring the level of SYBR Green I fluorescence. The background signal (relative fluorescence units (RFUs)) of SYBR Green I dye generated by the primer dimers or the hairpins was minimized with the use of Dabcyl-labeled primer, and the signal value decreased to 0 RFU (Fig. 2A), while primers without any modification generated about 3500 RFUs of SYBR Green I signal in contrast with blank control (0 RFUs) (Fig. 2B). Moreover, recombinant wild-type plasmid was used as reference for standard curve calculations. The sensitivity of quantification was increased 10-fold from 10^3 to 10^2 copies with the C_t value near 35 and $R^2 > 0.999$ (Fig. 3).

After thermal cycling, the parameter values for the analysis should be dye-specific. The baseline values for four fluorescent dyes (SYBR Green I, JOE, Cy3, and Cy5) were selected as automatic, and the threshold values of the dyes were adjusted differently, from 0.05 to 0.18, with SYBR Green I dye the highest and Cy3 the lowest. Therefore, the standard curves that correspond to the four dyes were calculated automatically, with the slope, intercept, and R^2 given (Fig. 3). PCR efficiency was confirmed by the automated method of serial dilutions and calculated from the slope of each curve. Fluorescent SYBR Green I generated the best result with a value of 99.9%, while Cy3 had the worst, 102.1%. The C_t values gained from Cy3 were greater than the others in each concentration.

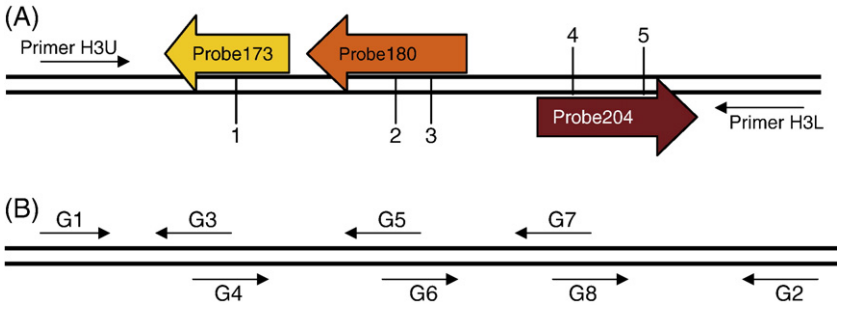

Fig. 1. Diagrammatic representation of the oligonucleotides used in this study. (A) Schematic representation of primers and hybridization probes indicating positions and orientations. 1, drug-resistant site rtV173L; 2, rtL180M; 3, rtA181V; 4, rtS202IG; 5, rtM204IV. (B) Schematic representation of primers used in the site-directed mutagenesis and gene assembly PCR, e.g., primer in group G1 with G2 generates fragment fused with product of primer G5 with G8 to construct a fragment at 743 bp, within the rt173 mutation. The primers corresponding to G1–G8 are listed in Table 5.

Table 1
Oligonucleotides used in HBV mutant detection

Target	Sequence 5′–3′	Position (nt)	Product size (bp)	Annealing temperature
H3U	Dabcyl–TGTATTCCCATCCCATCATCCT	599–620	164	54.5°C
H3L	GGCCCCCAATACCACATCA	762–744		55.1°C
173	JOE–TA**TGGGA**GTGGG–BHQ1	639–650		65°C
180	Cy3–CG**TTTCTCCT**GGC**T**C–BHQ2	659–673		66°C
204	Cy5–C**CATATAACT**GA**AA**–BHQ3	742–729		65°C

Boldface indicates the LNA monomer.

Sensitivity of the quadruplex quantification

The multiplex real-time PCR approach detected as few as 10^2 copies of templates as shown in Fig. 3 and Table 2, with $R^2 = 0.999$, at a reliable value of $C_t < 35$, the same as the single-plex PCR. The sensitivity for the wild-type HBV in this analytical technique was 10^2 copies (Fig. 4A). More than 1% mutants in a population of 10^8-10^4 copies of wild-type (rt173,

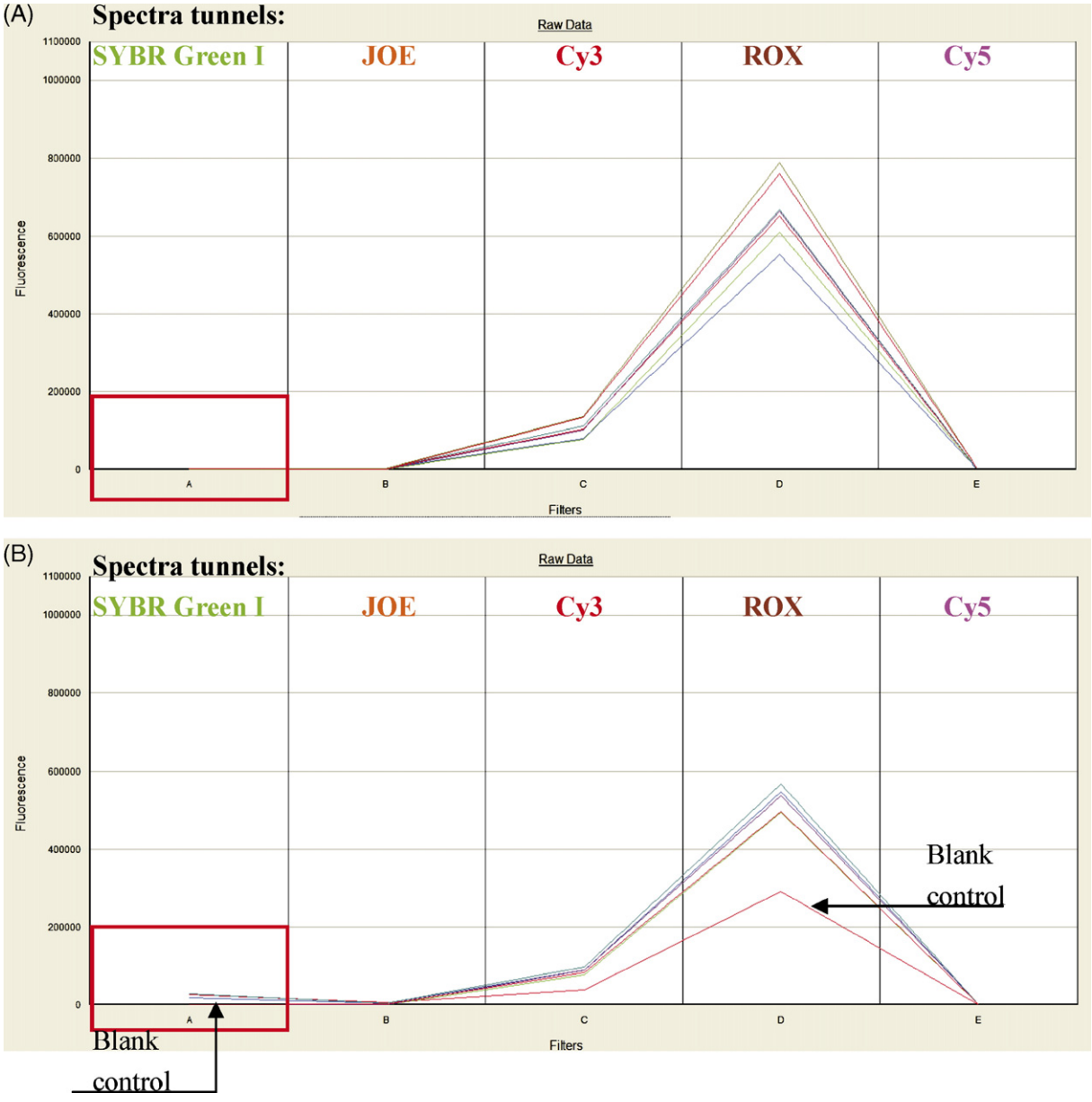

Fig. 2. The background signal (relative fluorescence units (RFUs)) of SYBR Green I dye generated by the primer dimers or the hairpins. All five fluorescent signals were collected at the first cycle. (A) Background signal of SYBR Green I dye was minimized with the use of Dabcyl-labeled primer, and the signal value decreased to 0 (red rectangle). (B) Primers without any modification generated about 3500 RFUs of SYBR Green I signal in contrast to blank control (0 RFUs) (red rectangle).

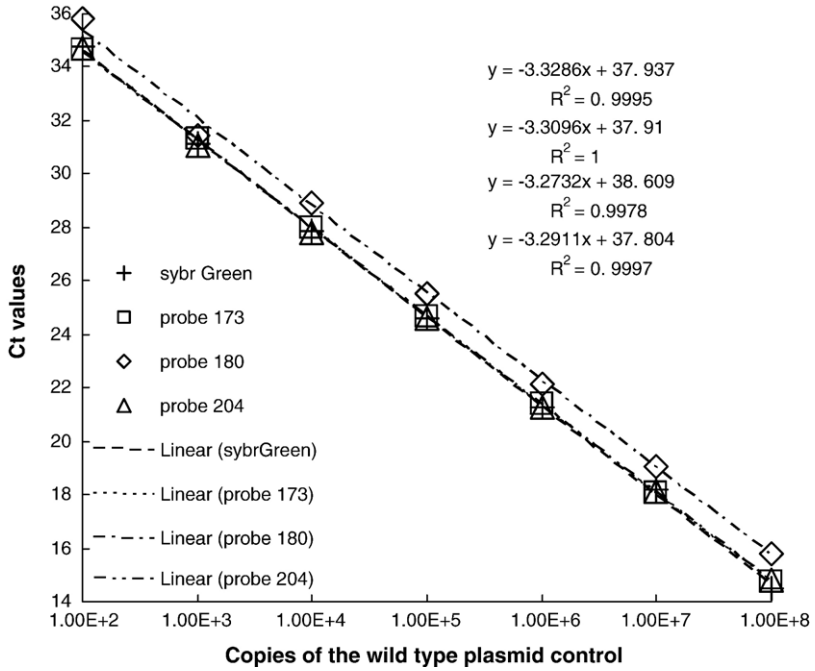

Fig. 3. Calculated curves based on four-color multiplex real-time PCR results of wild-type plasmid control from 10^2 to 10^8 copies with $R^2 > 0.99$. Equations in the upper right corner correspond to SYBR Green I dye, probe 173, probe 180, and probe 204 from the top down.

rt180/rt181, and rt202/rt204) could be identified (Figs. 4B–4D). The detection limit for the mutant type was derived from the equation

$$\text{total HBV} = \text{wild-type HBV} + \text{mutant-type HBV}.$$

From this equation the true detection limit of the mutant HBV was determined indirectly from the wild-type signal since our probes were designed to hybridize with the wild-type DNA sequence. The technique (results not shown) could detect as few as 75 copies of wild HBV ($R^2 = 0.98$); however, we chose 10^2 ($R^2 = 0.999$) to achieve a standard graphical interpretation of the data by regression analysis. As seen in Table 2, we also found that to distinguish the mutant-type HBV accurately from the wild-type and attain high sensitivity, the concentration of the mutant type in the samples had to exceed 100 copies. However, probe 180 had a one- to threefold lower sensitivity than the others (data not shown). All reactions were run in parallel.

Table 2

Total copies	Ratio of mutant (rt173 or rt180/181 or rt202/204) plasmid control: wild-type plasmid control								
10^8	**100:0**	99:1	95:5	50:50	25:75	10:90	5:95	1:99	0:100
10^7	**100:0**	99:1	95:5	50:50	25:75	10:90	5:95	1:99	0:100
10^6	**100:0**	99:1	95:5	50:50	25:75	10:90	5:95	1:99	0:100
10^5	**100:0**	99:1	95:5	50:50	25:75	10:90	5:95	1:99	0:100
10^4	**100:0**	99:1	95:5	50:50	25:75	10:90	5:95	*1:99*	0:100
10^3	**100:0**	**99:1**	95:5	50:50	25:75	*10:90*	***5:95***	***1:99***	0:100
10^2	**100:0**	**99:1**	**95:5**	50:50	***25:75***	***10:90***	***5:95***	***1:99***	0:100
NTC	**NTC**	**NTC**	NTC	**NTC**	**NTC**	**NTC**	**NTC**	**NTC**	**NTC**

Wild-type and mutant mixtures were used for sensitivity determination. The template for the mutant with the rt173 or rt180/181 or rt202/204 mutation was mixed with the template for the wild-type at a constant ratio in each dilution. NTC, no-template control; boldface, cannot be detected or distinguished by any reporter; boldface italic, detected but cannot be distinguished; italic, detected and poorly distinguished; underlined, poorly detected and cannot be distinguished; all others were well detected and distinguished by all reporters.

Hepatitis B surface antigen (HBsAg)-positive serum sample testing

Of the 30 HBsAg-positive samples, 3 (10%) were positive at the rt180 or rt181 mutation (group B, resistant to lamivudine, emtricitabine, or adefovir), 4 (13.33%) at the rt202 or rt204 mutation (group C, resistant to lamivudine, emtricitabine, or telbivudine), and 5 (16.67%) at both the rt180/rt181 and the rt202/rt204 mutation (group D, resistant to lamivudine, or emtricitabine, or adefovir with telbivudine) (Table 3). More than 10^5 copies of total viral DNA with >85% of mutants were screened in all isolates. Real-time PCR tracing plots are presented in Fig. 5. Genomic sequences of clinical isolates were confirmed by direct sequencing. The results showed that the mutant types were the same as the real-time PCR assay detected. The time required to perform this analysis consisted of 1 h for DNA isolation, 25 min for preparation of the reaction, and 2 h for the sample run. To achieve a balance between prevention of background signal (blank signal) and false positives, an empirical thermocycling parameter of 35 cycles was used.

Discussion

The emergence of HBV strains carrying mutants associated with drug resistance is probably a result of the nature of the

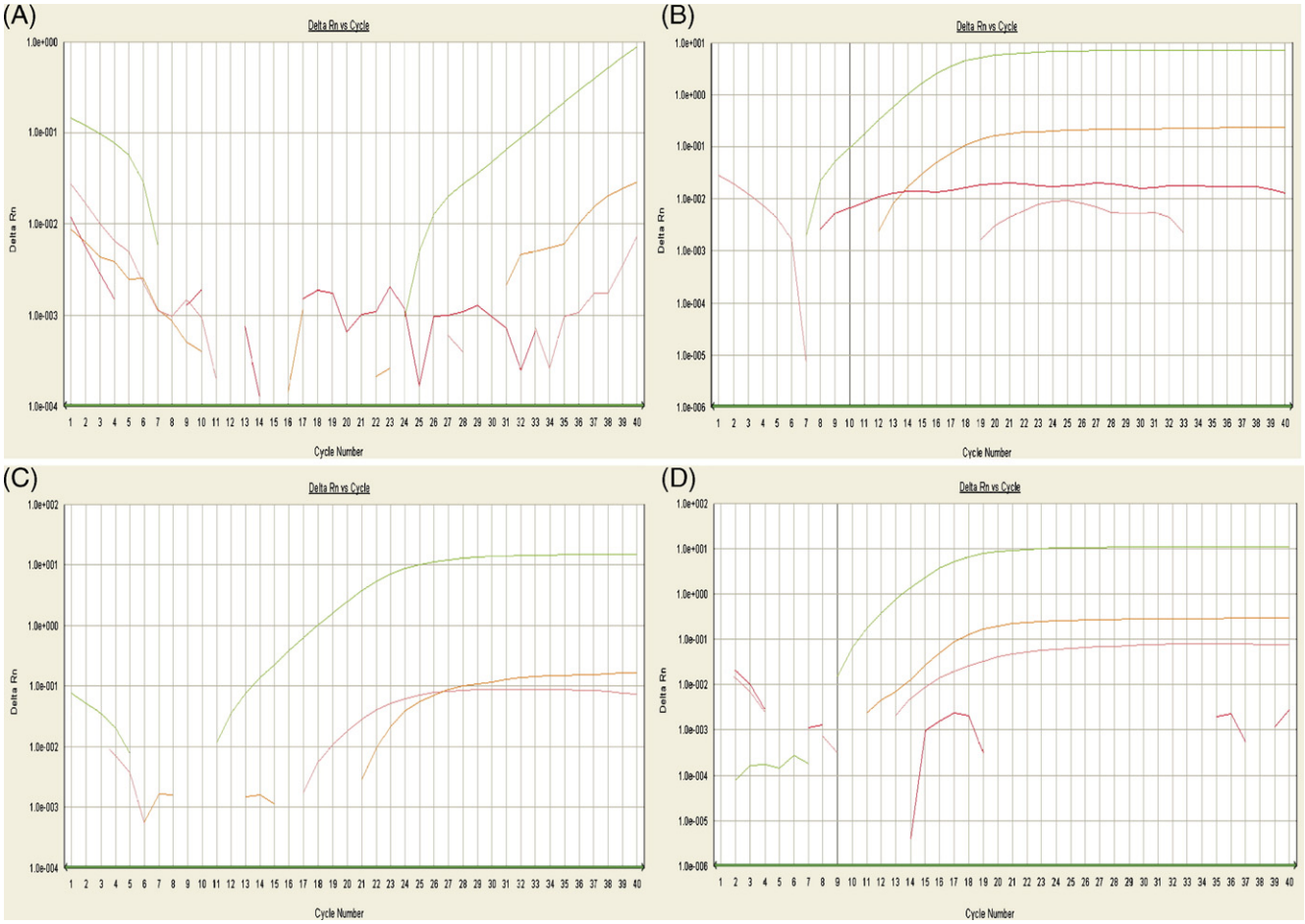

Fig. 4. The real-time PCR tracing plots (log scale) of parts of wild-type/mutant mixing experiments. Green line for SYBR Green I signal, orange for probe 173, red for probe 180, and pink for probe 204. (A) 10^2 copies of total viral DNA with full percentage of mutant plasmid control (ID 10). (B) 10^8 copies of total viral DNA with 99% of wild-type plasmid control and 1% of mutant plasmid (ID 10). (C) 10^7 copies of total viral DNA with 10% of mutant plasmid control (ID 2). (D) 10^7 copies of total viral DNA with full percentage of mutant plasmid control (ID 2).

replication process that occurs via an RNA intermediate requiring the activity of a reverse transcriptase [14]. This replication process lacks 3′ exonuclease proofreading activity, leading to misincorporation of bases. Natural mutants of HBV occur at a relatively high frequency, estimated to be 10^{-5} nt per year [15], compared to 10^{-9} nt per year for most other DNA viruses [16]. The high mutation and replication rate results in a viral population containing many variants with single mutations, many of which are capable of producing their own progeny. In addition, the nucleotide analogues, such as lamivudine, emtricitabine, adefovir, and telbivudine, are effective in inhibiting HBV replication during the viral DNA synthesis through chain termination [2–5]. Drug-resistant strains have been described after the therapeutic interventions of antiviral therapy, with the appearance of HBV mutations in the *polymerase* locus [6–8]. Thus, drug-resistant mutants probably exist in the viral population even prior to the start of antiviral therapy, albeit at a very low level [17]. In China, however, there is no wide clinical use of antiviral drugs as yet, with the exception of lamivudine; therefore virtually all clinical isolates are resistant to only lamivudine. The detection of the recognized and emerging HBV mutations is the key to understanding the natural history of HBV infections in infected individuals and the documentation of the epidemiological data. The studies facilitate clinicians and scientists by shedding more light on the responses to therapeutic and preventive interventions and overall management of HBV-related diseases.

Previous studies of real-time PCR in HBV mutant monitoring have shown the ability to detect minority quasispecies at a level of 1 in 1000 by using primers in which the relevant base pair mutation was at or near the terminal 3′ base [18] and also the highly sensitive and easily applicable assay INNO-LiPA [19]. Zhang et al. have shown that hybridization probes with melting curve analysis provided a reliable result, with 5% mutant among wild-type virus [20]. However, there is no multiple-site identification assay to date. This article describes a reliable, rapid, and sensitive method for quantifying virus DNA and identifying four nucleoside analogues associated antiviral/drug-resistance mutations in the *polymerase* gene of HBV.

To prevent hybridizing competition, the accumulation of PCR amplicons could be monitored by measurement of the level of SYBR Green I fluorescence. One disadvantage of this

Table 3
Clinical isolates with (positive) or without (negative) mutants

Sample	Mutant site			Group
	rt173	rt180/rt181	Rt202/rt204	
1	–	–	–	A
2	–	–	–	A
3	–	+	+	D
4	–	+	–	B
5	–	+	–	B
6	–	–	–	A
7	–	–	+	C
8	–	–	–	A
9	–	–	+	C
10	–	–	–	A
11	–	–	–	A
12	–	–	–	A
13	–	–	–	A
14	–	+	+	D
15	–	–	–	A
16	–	–	–	A
17	–	–	–	A
18	–	–	–	A
19	–	+	+	D
20	–	+	+	D
21	–	+	–	B
22	–	–	–	A
23	–	–	–	A
24	–	–	–	A
25	–	+	+	D
26	–	–	–	A
27	–	–	–	A
28	–	–	+	C
29	–	–	+	C
30	–	–	–	A

+, positive; –, negative; A, no resistance; B, resistant to lamivudine, emtricitabine, or adefovir; C, resistant to lamivudine, emtricitabine, or telbivudine; D, resistant to lamivudine, or emtricitabine, or adefovir with telbivudine.

method is, however, that both primer dimers and hairpins can raise the fluorescence signal of SYBR Green I, limiting sensitivity. To solve this drawback, we developed a novel method that attached the Dabcyl dye to the 5′-terminus of the sense primer, which has more secondary structure. The fluorescence of stain molecules that bind to the primer or its dimer was quenched effectively. The chromogenic molecules that bind at sites sufficiently remote from the quencher exhibit fluorescence with chain elongation. Thus, the signal in amplicon measurements more accurately indicated initial target numbers, and the background fluorescence was reduced [21]. Meanwhile, JOE, Cy3, and Cy5 reported three identifying signals when probes matched with specific DNA sequences within the wild-type strains. The combination of these five fluorescence dyes could minimize the interference of fluorescence resonance energy transfer with separated ex \em filters of the 7500 system according to their spectra.

According to the fluorescence raw data (not shown), probes 173 and 204 were as sensitive as the SYBR Green I dye within different thresholds, but probe 180 was less so. This suggests that the target strands formed a secondary structure thereby preventing hybridization to ssDNA since it was only seven nucleotides from probe 173 to probe 180. Probe 204 also gave a low signal when hybridized on the same strand as the other probes; this may not be due to the structure prevention as described by Jacobsen et al. [22] but to the decreasing 5′ exonuclease activity of *Taq* polymerase and the thermal dynamics block. Thus, we have chosen SYBR Green I dye instead of another probe and, furthermore, used probe 204 to hybridize on the same template as the antisense primer, which generated a reliable result.

LNA probes were 12–15 nt, with the same T_m and quench efficiency as the TaqMan MGB but a single LNA mismatch causing decreases in T_m values ranging from 12 (G/T) to 30°C (A/A) [23]. The use of LNA probes results in an increased melting temperature with a simultaneous increase in the ΔT_m between the perfectly matched and the mismatched targets (data not shown), which made it possible to detect single base substitutions. The precision of management of the annealing temperature in the 9600 emulation of the 7500 system was sufficient to enable us to distinguish samples differing by a single base. However, since many nucleotide sequence variations occurred at the same site of probe binding, it was not always possible to differentiate the genotype of any particular virus with the probe alone.

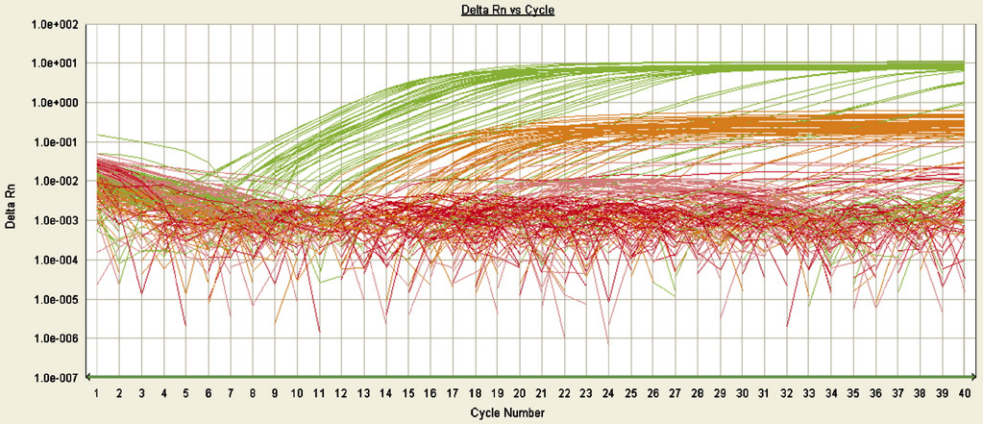

Fig. 5. The real-time PCR tracing plots (log scale) of 30 HBsAg-positive clinical isolates. Green line for SYBR Green I signal, orange for probe 173, red for probe 180, and pink for probe 204.

Table 4
Variants associated with drug resistance corresponding to the amino acid and DNA codon

Nucleic acid analogue	Amino acids reverse transcriptase				
	V173**L**	L180**M**	A181**V**	S202**IG**	M204**IV**
Adefovir			+		
Emtricitabine	+	+			+
Lamivudine	+	+			+
Telbivudine					+
DNA codons					
Wild-type	GTN	CTN	GCN	TCN AGY	ATG
Mutant	**C**TN **T**TR	**A**TG	G**T**N	**G**GN A**T**H	AT**H** **G**TN

Boldface indicates the mutant site. + indicates that the variant was associated with the drug resistance. N indicates A or T or C or G, R indicates A or G, H indicates A or T or C, Y indicates C or T.

Moreover, it should be noted that the findings indicate that our method is suitable for point mutation detection. However, it remains to be determined whether the method is also suitable for other clinically relevant mutations, such as deletions and insertions within the viral genome.

This study shows that real-time PCR with SYBR Green I dye is a reliable, rapid, and convenient technique for the quantification of HBV mutants. Furthermore, by incorporating fluorescent LNA probes, this technique becomes handy in identifying and classifying mutations in the *polymerase* gene. Being sensitive, specific, accurate, rapid, and convenient in nature, this technique could be a suitable diagnostic tool with wide application particularly in cases in which large volumes of clinical samples are handled. Furthermore, clinical application of this analytical method could tremendously improve the management of patients with HBV-induced diseases. The technique could also be handy in HBV genotyping.

Materials and methods

Drug-resistant mutant sites of the HBV *polymerase* region are listed in Table 4 according to previous publications [6–8,24].

Design of PCR primers and LNA probes

The sequences of primers and LNA probes used in this study are listed in Tables 1 and 5. Schematic representations of the locations and orientations of the oligonucleotides used are shown in Figs. 1A and 1B. Primers and hybridization probes were designed based on the nucleotide sequences of wild-type HBV (GenBank Accession No. NC_003977 with nt 735 C changed to T) on the basis that the wild-type HBV conserved sequence would increase the sensitivity and specificity of our technique. Moreover, position 735 has a T, regardless of the HBV type, in over 90% of the HBV sequences we analyzed from GenBank. The probes containing LNA residues were designed such that the mutation site was placed at a central location within the sequence of the oligonucleotide, with a T_m of 65-66°C. The length was between 12 and 15 nucleotides with the mutation site replaced by an LNA residue. The oligonucleotide probes contained between 4 and 8 LNA residues, which were introduced within the probe with a separation of 1–3 nucleotides. The LNA probes were synthesized and labeled by Proligo Co., Ltd. (Singapore). The other oligonucleotides were synthesized and labeled by TaKaRa Biotechnology (Dalian) Co., Ltd. (China). The sequences of all the oligonucleotides were analyzed for the absence of false priming sites and formation of primers, probes, primer/probe hybrids, and secondary structures and confirmed with the aid of the Lasergene sequence analysis software (DNAStar, Inc., Madison, WI, USA).

Extraction of HBV DNA from sera

Thirty HBsAg-positive sera, uncharacterized for HBV mutants, were obtained from First Affiliated Hospital of Zhejiang University. Code numbers were used instead during this investigation. Permission to test the samples was obtained from the Zhejiang University Conjoint Ethics Committee.

HBV DNA was extracted from serum samples (100 μl) using the QIAquick viral DNA extraction kit (Qiagen GmbH, Hilden, Germany). The DNA was eluted in 100 μl of sterile dH_2O and stored at −20°C until assayed.

Table 5
Oligonucleotides used in the site-directed mutagenesis and gene-assembly PCR

Primer	Sequence 5′–3′	Position (nt)	Product size (bp)	Group
ACU	TGTTGCCCGTTTGTCCTCTACTTC	462–485	743	G1
ACL	CCGTGGGGGTTGCGTCAG	1204–1187		G2
173ML	GGCCCA**R**TCCCATAGGAATCTT	652–631	With ACU 191	G3
173U	TGGGCCTCAGTCCGTTTCTC	647–666	With ACL 558	G4
180ML	CTGAGCCA**T**GAGAAACGGACTGA	675–653	With ACU 214	G5
181ML	CTGA**A**CCAGGAGAAACGGACTGA	675–653	With ACU 214	G5
180181ML	CTGA**A**CCA**T**GAGAAACGGACTGA	675–653	With ACU 214	G5
180MU	GCTCAGTTTACTAGTGCCATTTGTTCA	670–696	With ACL 535	G6
181-180181MU	G**T**TCA GTTTACTAGTGCCATTTGTTCA	670–696	With ACL 535	G6
202ML1	ATAGC**C**GAAAGCCAAACAGTGGG	738–716	With ACU 277	G7
204ML	ATAGCTGAAAGCCAAACAGTGGG	738–716	With ACU 277	G7
202ML2-202204	ATAG**A**T GAAAGCCAAACAGTGGG	738–716	With ACU 277	G7
202MU1	C**GG**CTATATGGATGATGTGGTATTGGGG	732–759	With ACL 473	G8
202MU2	CA**T**CTATATGGATGATGTGGTATTGGGG	732–759	With ACL 473	G8
204MU1	CAGCTAT**G**TGGATGATGTGGTATTGGGG	732–759	With ACL 473	G8
204MU2	CAGCTATAT**H**GATGATGTGGTATTGGGG	732–759	With ACL 473	G8
202204MU1	CA**T**CTATAT**H**GATGATGTGGTATTGGGG	732–759	With ACL 473	G8
202204MU2	CA**T**CTAT**G**TGGATGATGTGGTATTGGGG	732–759	With ACL 473	G8

Boldface indicates the mutant site. H indicates A or T or C, R indicates A or G. Underline indicates complementary terminal regions.

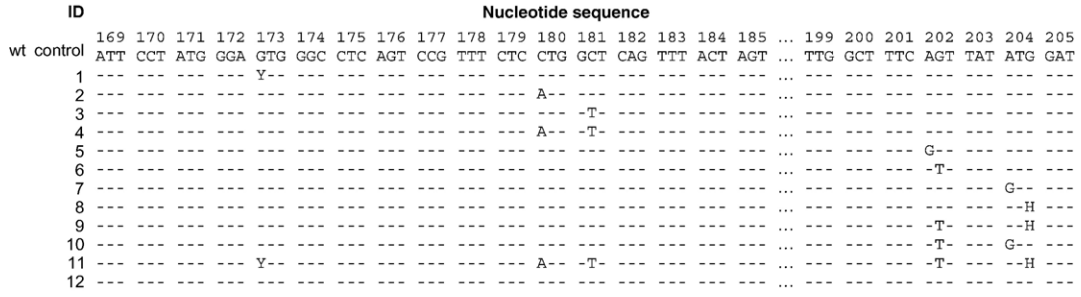

Fig. 6. Detailed presentation of the recombinant HBV reference plasmid panel. Codon numbering is according to genotype A reverse transcriptase numbering. Three periods indicate a gap introduced between codons 185 and 199; sequences in this gap are not relevant for the design of the assay. ID, clone identification number; wt, wild type; H, A or T or C; Y, C or T.

Construction of the recombinant plasmids

Recombinant plasmids containing inserts spanning the target region and specific single-base mutations were constructed by overlap extension PCR. Primers in groups G1/G3 and G2/G4, G1/G5 and G2/G6, as well as G1/G7 and G2/G8 (Fig. 1B and Table 5) were used to amplify three clusters of corresponding fragments for subsequent splicing by overlap extension PCR to produce fragments of 743 bp for A-T cloning. The primers described previously were designed to generate site-directed mutagenesis so that all corresponding fragments contained a single-base substitution of rt173, rt180 and rt181, or rt202 and rt204. The DNA fragments were purified and cloned into the pMD18-T vector (T-A cloning kit; TaKaRa Biotechnology (Dalian) Co., Ltd.). The transformation of *Escherichia coli* with the recombinant plasmids was carried out as instructed by the manufacturer (TaKaRa Biotechnology (Dalian) Co., Ltd.). The plasmids were extracted and purified by standard methods and confirmed using an ABI Prism 3730 sequencer (Invitrogen Biotechnology (Shanghai) Co., Ltd., China) (Fig. 6). The DNA concentration was determined with a spectrophotometer at 260 nm, and the corresponding copy number of plasmids was calculated. Plasmid serial dilutions ranging from 1×10^8 copies to 1 copy were then prepared.

Real-time PCR optimization

Plasmid controls were used as templates in this PCR assay to evaluate the method and then followed with sera samples testing. The real-time PCR was performed in a total volume of 30 μl containing 1 μl of DNA template, 0.3 μl of 1:1000 × SYBR Green I and ROX dye (Molecular Probes, Inc., Eugene, OR, USA), 1.5 U of *Ex-Taq* polymerase with 3 μl of $10 \times Mg^{2+}$-free buffer (TaKaRa Biotechnology (Dalian) Co., Ltd.), 6 mM Mg^{2+}, 0.2 mM dNTPs, 9 pmol of each primer, and 15 pmol of each probe. The 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) performs product detection by monitoring fluorescence changes in spectral windows with wavelengths ranging from 520 to 675 nm. Thermocycling parameters were as follows: 95°C for 20 s; then 40 cycles of 88°C for 10 s, 59°C for 10 s, and 72°C for 40 s to measure the fluorescence signal; followed by the dissociation stage. Melting curves and quantitative analysis of the data were performed using the 7500 System SDS software v1.2.3 (Applied Biosystems). To determine the sensitivity of the detection system for mutant HBV DNA among wild-type HBV DNA, mutant and wild-type plasmid control DNAs were mixed at a constant ratio as shown in Table 2. Full percentage wild-type plasmid control DNAs were used as references for standard curve calculation in this sensitivity testing. SYBR Green I signal was used to generate the curve to quantify the total viral DNA level. LNA probe signals were used for establishing standard curves for wild-type viral DNA calculations according to the three mutation sites. Moreover, the quenching of the LNA probes' fluorescence was used to distinguish the LNA probes as to whether they were hybridized to the amplified viral DNA fragment (wild-type) or not hybridized (mutant).

During the clinical sample testing, wild-type plasmid control DNAs, diluted from 10^8 to 10^2 copies, were also used as references for standard curve calculation in the procedure. The standard curve for total viral DNA quantification was established according to the SYBR Green I signal, and LNA probe signals were used for establishing standard curves for wild-type viral DNA level calculations for the three mutation sites. Thus, the mutant viral DNA level was calculated according to the equation given above. Therefore, the mutant/total viral DNA ratio was calculated by comparing the mutant level to the total DNA level. In addition, no-template control and blank control were also presented for negative controls.

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